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Addition of exogenous polypeptides on the mammalian reovirus outer capsid using reverse genetics

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Addition of exogenous peptide sequences on viral capsids is a powerful approach to study the process of viral infection or to retarget viruses toward defined cell types. Until recently, it was not possible to manipulate the genome of mammalian reovirus and this was an obstacle to the addition of exogenous sequence tags onto the capsid of a replicating virus. This obstacle has now been overcome by the availability of the plasmid-based reverse genetics system. In the present study, reverse genetics was used to introduce different exogenous peptides, up to 40 amino acids long, at the carboxyl-terminal end of the σ1 outer capsid protein. The tagged viruses obtained were infectious, produce plaques of similar size, and could be easily propagated at high titers. However, attempts to introduce a 750 nucleotides-long sequence failed, even when it was added after the stop codon, suggesting a possible size limitation at the nucleic acid level.

1. Introduction

Mammalian reovirus under the trademark "Reolysin®" (derived from reovirus serotype 3 Dearing: T3/Human/Ohio/Dearing/55) is presently under study as an "oncolytic" virus in various clinical assays for the treatment of human cancers (recently reviewed in: Kelly et al., 2009; Lal et al., 2009; Thirukkumaran and Morris, 2009; Harrington et al., 2010). Reovirus, is among the few viruses that can be used as an oncolytic agent without prior genetic engineering. Reovirus is only weakly pathogenic in human adults and its replication is blocked in "normal" cells while it is able to replicate in, propagate, and destroy transformed/cancer cells; this is apparently due to the presence of an activated Ras oncogenic pathway in these cells. Direct or indirect activation of the Ras signaling pathway appears to increase viral uncoating, repress innate immunity and/or augment viral-induced apoptosis, resulting in the natural oncolytic activity of the virus (Alain et al., 2007; Marcato et al., 2007; Rudd and Lemay, 2005; Shmulevitz et al., 2010; Smakman et al., 2005; Strong et al., 1998).

The reovirus capsid is made of two concentric layers of proteins; the trimeric σ1 protein is exposed at the surface of the virion and binds onto target cells; the carboxyl-terminal globular domain being most exposed and responsible for high-affinity binding to the main cellular receptor, JAM-A. Reovirus virions enter cells by endocytosis and are partially uncoated before being released to the cytoplasm; in contrast, extracellular proteases can partially uncoat the virions to infectious subviral particles (ISVPs) that still harbor σ1 at their surface but that directly cross the plasma membrane to enter the cytoplasm where viral replication ensues (Danthi et al., 2010).

Despite its natural oncolytic ability, it is likely that reovirus binding onto normal cells via ubiquitous receptors such as sialic acid, JAM-A, and other unidentified receptor(s) (Antar et al., 2009; Coyne, 2009) will limit efficiency of the virus to reach some target cells in the organism. There are also some evidence to suggest that the virus receptors on the cell surface are either absent, or "masked" in some human cancer cell types, such as colorectal cancer cells (van Houdt et al., 2008). Finally, even though the virus appears to be only weakly pathogenic in humans, it cannot be excluded totally that some individuals, especially younger or immunodeficient patients, could develop disease due to reovirus inoculation, especially after systemic (intravenous) administration. Although its involvement in human biliary atresia of the newborn remains to be proven more conclusively, a significantly higher prevalence of reovirus RNA was detected in hepatic tissues from diseased individuals compared to normal controls (Tyler et al., 1998). Given strains of reovirus were also shown to induce liver pathologies, that are reminiscent of biliary atresia, in a murine experimental model (Parashar et al., 1992; Forrest and Dermody, 2003). The virus was also shown to induce "black foot syndrome" in immunodeficient SCID/NOD mice (Loken et al., 2004). There are thus possibly important benefits in the development of reovirus strains that are either attenuated (Kim et al., 2011) or targeted to bind specifically onto given cancer cell types, as proposed by others (Van Den Wollenberg et al., 2009). This

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last approach is currently under study for various oncolytic viruses such as adenovirus, measles virus, and herpesvirus, among others (Blechacz and Russell, 2008; Mathis et al., 2005; Campadelli-Fiume et al., 2011).

Until recently, it was not possible to modify the genome of reovirus, using the so-called "reverse genetics" approach that has been so widely used to study viruses belonging to most virus families. Nevertheless, a small 6-amino acids long peptide was added to the extreme carboxyl-terminal end of σ1 using a complex experimental approach in which cells are transfected with the plasmid encoding the tagged protein and then infected with a wild type virus to select recombinant viruses (van den Wollenberg et al., 2008). This strategy appears quite complex and requires an adequate cell line for the selection procedure.

A plasmid-based reverse genetics system was more recently described for mammalian reovirus by the group of Dr Terence Dermody (Kobayashi et al., 2007; reviewed by: Lemay, 2011); this novel approach was used by this group to study various reovirus mutants obtained by site-directed mutagenesis (see for example: Boehme et al., 2009; Danthi et al., 2008a,b; Kobayashi et al., 2007, 2009)), or to substitute a whole gene segment from one reovirus serotype to the other (Kobayashi et al., 2007; Zurney et al., 2009).

In the present study, the usefulness of the plasmid-based reverse genetics approach was further established by adding different epitope tags at the carboxyl-terminal end of σ1. Viruses harboring up to a 40 amino acids long peptide were readily recovered. However, infectious viruses could not be recovered when the sequence encoding the yellow fluorescent protein was fused to σ1, even when a stop codon was introduced to avoid the production of a fusion protein. This suggests the possibility of a size limitation of exogenous sequences that can be added at the nucleic acid level, independently of protein structure or function.

2. Materials and methods

2.1. Cells and viruses

L929 cells and Vero cells were originally obtained from the American Type Culture Collection and were used for reovirus propagation and titration; BHK21 cells stably expressing the T7 RNA polymerase (Buchholz, Finke, and Conzelmann, 1999) were a generous gift from the laboratory of Dr. John Hiscott (Lady Davis Research Institute, Montréal, Canada). All these cells were grown in minimal Eagle medium (MEM; Wisent) containing 5% fetal bovine serum (FBS Gold, PAA laboratories).

2.2. Antibodies

Hybridoma cell lines producing either anti-σ3 (4F2) or anti-μ1 (10F6) have been described before (Virgin et al., 1991) and were obtained from Dr. Kevin Coombs (Department of Microbiology, Manitoba University). These cells were grown in MEM for suspension culture with 10% fetal bovine serum, proline (20 μg/ml) and β-mercaptoethanol (50 μM); they were fed every 2 or 3 days to obtain cell concentration between 2×10^5 and 5×10^5/ml, they were resuspended in fresh medium at a concentration of 5×10^5 cells/ml and left until most cells were dead as judged by trypan blue exclusion; cells and debris were removed by centrifugation at low speed and supernatants containing the monoclonal antibodies were kept at 4 °C with 1 mM sodium azide as preservative. The polyclonal antiserum directed against the carboxyl-terminal head domain of σ1 was produced originally in the laboratory of Dr. Terence Dermody (Vanderbilt University, Tennessee) and was a generous gift from Dr. Earl Brown (University of Ottawa). The mouse anti-polyhistidine monoclonal antibody was obtained from Qiagen (Qiagen, Mississauga, Ontario, mouse Penta-His antibody, Catalog #34660). The mouse anti-HA tag (12CA5) was from Berkeley Antibody Company (BabCO) and a generous gift from the laboratory of Dr. Pierre Belhumeur (Département de Microbiologie et Immunologie, Université de Montréal). The rabbit anti-tubulin was obtained from ICN Biomedicals Inc.

2.3. Plasmid constructs

The plasmids harboring separately each of the cDNA corresponding to the 10 genes of reovirus serotype 3 Dearing, under the transcriptional control of the T7 promoter, have been described (Kobayashi et al., 2007) and were obtained from the laboratory of Dr. Terence Dermody (Vanderbilt University, Nashville, Tennessee). Medium scale plasmid DNA was column-purified using Qiagen plasmid midi kit and endotoxin-free buffers (Catalog #12243 and #19048), as recommended by the manufacturer. Concentration and purity were determined by measuring optical density using a nanodrop microspectrophotometer (ND-1000, Thermo Scientific). To insert sequence encoding for the hexahistidine or single HA tag at the end of the σ1-encoding sequence, the corresponding plasmid was submitted to site-directed mutagenesis using the QuickChange® Lightning Site-Directed Mutagenesis Kit (Stratagene, Catalog #200518), as recommended by the manufacturer. In an additional construct, a unique *Spe*I site was introduced following the hexahistidine tag, just before the stop codon, thus adding two amino acids to the protein (a threonine and a serine); this was also done by site-directed mutagenesis using the plasmid encoding hexahistidine-tagged σ1. This same plasmid was used to introduce 3 additional copies of the HA tag. The pMPY-3xHA plasmid (Schneider et al., 1995) was used as a template for PCR amplification of the 3HA-encoding region using oligonucleotides designed to introduce the *Spe*I site at both ends of the PCR product that was cloned thereafter at the *Spe*I site following the hexahistidine tag. The sequence encoding the enhanced yellow fluorescent protein was amplified from the pcDNA.1.eYFP.MCS(MB) plasmid (a generous gift from Dr. Jacques Thibodeau) and also subcloned at the *SpeI* site. In a final construct, a stop codon was introduced by site-directed mutagenesis 3 amino acids after the beginning of the YFP-encoding sequence. The presence of the expected mutations or insertions, and absence of other changes in the adjacent region, was verified by sequencing the final plasmids on small-scale preparation of endotoxin-free plasmid DNA that was column-purified, as recommended by the manufacturer (Zyppy plasmid miniprep kit, Zymo Research). The different oligonucleotides used as primers for mutagenesis, PCR and sequencing are presented in Table 1.

2.4. Recovery of infectious viruses by plasmid-based reverse genetics

Recovery of infectious reovirus stocks was done using a modification of the original procedure (Kobayashi et al., 2007). The nine wild-type plasmids, prepared as medium scale preparation, were simultaneously introduced with the plasmid encoding the tagged σ1; transfection was done using Fugene 6 (Roche), as recommended by the manufacturer. Approximately 0.10 μg of each plasmid was used to transfect semi-confluent 35 mm-diameter petri dish of BHK21 cells that express the T7 RNA polymerase. The medium was recovered 3–4 days later, cells were trypsinized and plated in a 100 mm-diameter petri dish with the medium recovered previously and completed with 9 ml of complete medium containing 5% heat-inactivated fetal bovine serum. Two-third of the medium was replaced with fresh medium, 3 days later, and incubated for another 4 days before being frozen at −80 °C and used as starting virus stocks. Virus clones were recovered from single plaques obtained on Vero cells in the presence of chymotrypsin;